—Original Articles—

The self-renewal process of murine hemopoietic stem cells supported by interleukin-3 and the synergistic factors and the probability of its occurrence

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Summary

By use of the in vitro murine blast cell colony (Bl) assay system, Bl-constituting cells supported by interleukin-3 (IL-3), IL-3 + interleukin-6 (IL-6), IL-3 + granulocyte colony-stimulating factor (G-CSF) and IL-3 + interleukin-1 (IL-1) were replated and the frequencies of secondary (2nd) granulocyte/erythrocyte/macrophage/megakaryocyte (GEMM) colony and Bl progenitors were examined. According to the statistical method reported by Nakahata et al, the p values for the hemopoietic stem cells to self-renew were calculated and all cytokine groups produced similar p values ranging between 0.576 and 0.596. Further studies using IL-3-supported and IL-3 + G-CSF-supported Bl showed that the 2nd Bl progenitors could be produced even when there were more than 150 primary Bl-constituting cells per colony in the case of IL-3 + G-CSF, but no 2nd Bl progenitors were found in the case of IL-3. Their appearance was limited within the smaller primary Bl when supported by IL-3. Again there was a difference in the net product number of 2nd Bl progenitors, that is, the addition of G-CSF to the primary culture could produce around double the number of 2nd Bl progenitors. These data led us to hypothesize that synergistic factors could not modify the self-renewal probability, but maintained the self-renewal process for a longer period, in other words, for several cellular divisions.

Key words: hemopoietic stem cell, self-renew, blast cell colony, interleukin-3 (IL-3), granulocyte colony-stimulating factor (G-CSF)

Introduction

A number of hemopoietic cytokines have been identified, and molecular biology techniques can produce large amounts of cytokines, making it possible to use these cytokines for in vivo administrations. Erythropoietin (Epo) and granulocyte colony-stimulating factor (G-CSF) have already been used clinically on several anemic and neutropenic patients and their therapeutic value has been established. A lot of scientists and clinicians have been interested in the cytokines regulating hemopoietic stem cells, and synergistic factors such as

interleukin-6 (IL-6), G-CSF, interleukin-1 (IL-1), interleukin-11 (IL-11). The stem cell factor (SCF) has also been energetically investigated to expand stem cells both in vitro and in vivo^{1~4)}.

The hemopoietic stem cells are few in number, and are present in the hemopoietic organs; they possess unique biological characteristics such as multipotentiality, dormancy in the cell cycle and self-renewability^{5,6)}. Nakahata et al have studied their characteristics closely by use of their established BI assay system, and have proposed a so-called stochastic model of their proliferation and differentiation processes⁷⁾. By use of replating experiments with BI-constituting cells, they also calculated the p value for stem cells to self-renew and noted it to be around 0.589⁸⁾. How to expand the hemopoietic stem cells is a difficult question and it is important first to understand the biological events which occur during the self-renewal process.

In this study, we tried to find out whether the hemopoietic cytokines, especially so-called synergistic factors, could modify the self-renewal process of the hemopoietic stem cells.

Materials and Methods

(1) Cell preparations

Eight to 12-week-old female BDF1 mice were purchased from Japan SLC (Shizuoka, Japan). 5-FU (Kyowa Hakko Kogyo, Tokyo, Japan) at 150 mg/kg body weight was administered through the tail vein 4 days before the sacrifice of the mice. Spleen cells were then harvested and single cell suspensions were prepared from the pooled spleens of three mice.

(2) Growth factors and clonal cell culture

Silkworm-derived recombinant murine IL-3 with a specific activity of 2.8×10^8 U/mg protein was donated by Dr. A. Miyajima (DNAX, Palo Alto, CA)⁹⁾. Recombinant human IL-6 with a specific activity of 5×10^6 U/mg protein was donated by Ajinomoto Co. Inc. (Kawasaki, Japan)¹⁰⁾. Purified recombinant human G-CSF expressed in CHO cells was a gift from Chugai Pharmaceutical Co. (Tokyo, Japan). Recombinant human IL-1 β expressed in *E. coli* was generously donated by Otsuka Pharmaceutical Co. (Osaka, Japan). CHO-derived human recombinant erythropoietin (Epo) was provided by Chugai Pharmaceutical Co. WEHI-3 conditioned medium (WEHI-3 CM) was a gift from Dr. T. Suzuki (Kirin Brewery Co., Gunma, Japan). The concentrations of the factors in this study were as follows: IL-3, 200 U/ml; IL-6, 100 ng/ml; G-CSF, 10 ng/ml; IL-1 β , 2 ng/ml; Epo, 2 U/ml.

Methylcellulose culture was performed in 35 mm Falcon suspension culture dishes (Beckton Dickinson Labware, Lincoln Park, NJ). One ml of culture contained 1×10^6 spleen cells of 5-FU treated mice, alpha-medium (Sigma Chemical Co., St. Louis, MO), 1.2% 1500 cps methylcellulose (Wako Pure Chemical Industries, Osaka, Japan), 30% fetal calf serum (FCS) (Hyclone Laboratories, Inc., Logan, UT), 1% deionized fraction V bovine serum albumin (BSA) (Sigma), 1×10^{-4} mol/L 2-mercaptoethanol (Sigma) and hemopoietic factors. Dishes were incubated at 37°C in a humidified atmosphere flushed with 5% CO₂. Colony types were determined on the specified culture day by in situ observation with an inverted microscope according to the criteria described previously⁷⁾. Abbreviations for colony types

are as follows: GM, granulocyte/macrophage; GEMM, granulocyte/erythrocyte/macrophage/megakaryocyte; and Bl, blast cell colonies.

(3) Replating experiments

On day 5-10 of culture, small Bls were individually lifted with an Eppendorf micropipette, and suspended in 0.1 ml α -medium. After gentle pipetting samples were added individually to 0.9 ml methylcellulose culture dishes containing WEHI-3 CM (10% v/v) and Epo. The dishes were then incubated at 37°C in a humidified atmosphere flushed with 5% CO₂. Colony types were determined in the same manner as in the primary cultures.

(4) Calculation of self-renewal probability

Multipotential Bl were grouped into two; one produced both 2nd GEMM and Bl and the other produced only 2nd GEMM without Bl. If the Bl progenitor chose the self-renewal process, a "birth" process, the Bl-constituting cells should contain the 2nd Bl progenitors. On the contrary, if the stem cell chose a "death" process, 2nd Bl progenitors could no longer be produced. The latter choice was also called the "extinction" process and the extinction probability, designated as "s", was calculated as the ratio of multipotential Bl without 2nd Bl progenitors per all multipotential Bl. Following Harris' procedure¹¹⁾, the pvalue, the probability that a Bl progenitor at any given generation produces a new identical progenitor cell was calculated as follows: $p = (1 - \sqrt{s}) / (1 - s)$.

Results

1. Replating experiments with Bl supported by IL-3, IL-3 + IL-6, IL-3 + G-CSF, IL-3 + IL-1 β

Spleen cells of 5-FU-treated mice were cultured in the presence of IL-3, IL-3 + IL-6, IL-3 + G-CSF, and IL-3 + IL-1 β . Since spleen cells usually contained relatively higher number of multipotential progenitors than bone marrow cells did. From day 5 to 10 of culture, new Bl with less than 150 cells per colony appeared daily and were lifted by Eppendorf micropipette, and replated. In another experiments, morphological confirmation of constituent cells of Bl had already performed, and all of these cells revealed immature nuclei and absence of cytoplasmic differentiation. Secondary colonies were counted on day 12. Data from replating experiments of primary Bl are shown in **Table 1**. The constituent cells of Bl possessed the same biological characteristics as the hemopoietic progenitors and could make secondary colonies in the methylcellulose culture. Among them, most colonies were oligopotential GM colonies and multipotential GEMM colonies constituted of 5,000-100,000 cells. However, some colonies were a diffuse type and contained a small number of cells, ranging between 30 and 150, which looked like immature blast cells. Higher than 100% replating efficiencies seen in some colonies are probably due to technical errors in estimation of cell numbers.

Since the primary Bl were derived from 5-FU-resistant, dormant hemopoietic stem cells, the 2nd Bl that were witnessed in this experiment were probably derived from the dormant stem cells produced by the primary stem cells after cell divisions. Nakahata and Ogawa⁷⁾ reported the presence of both the primary Bl and the 2nd Bl, and the mechanism of the

Table 1 Results of replating experiments on blast cell colonies

(1) IL-3						(2) IL-3+IL-6					
Blast cell colony size	Number of secondary colonies				Replating efficiency(%)	Blast cell colony size	Number of secondary colonies				Replating efficiency(%)
	GM	GEMM	bl	Total			GM	GEMM	bl	Total	
55	12	3		15	27.3	47	2	1		3	6.4
35	14	2	2	18	51.4	33	10	1		11	33.3
30	21	1		22	73.3	45	26	6	1	33	73.3
61	40	8		48	78.7	68	43	4	1	48	70.6
46	23	10		33	71.7	45	14	2		16	35.6
44	21	5		26	59.1	30	4	1		5	16.7
41	24	6		30	73.2	36	12	4	4	20	55.6
48	15	3		18	37.5	65	35	3		38	58.5
64	32	11	1	44	68.8	38	11	3	2	16	42.1
105	33	4		37	35.2	41	2	2		4	9.8
51	21	6	2	29	56.9	44	19	7		26	59.1
48	10	3	2	15	31.3	84	72	9	1	82	97.6
44	12	11	3	26	59.1	87	27	2		29	33.3
48	23	5		28	58.3	77	49	6		55	71.4
30	5	2		7	23.3	72	48	6	4	58	80.6
55	9	4	1	14	25.5	60	30	3	4	37	61.7
30	10	3	3	16	53.3	78	25	14		39	50.0
71	9	8	9	26	36.6	81	43	1	1	45	55.6
86	19	3		22	25.6	125	48	14		62	36.8
116	35	3		38	32.8	100	48	14		62	62.0
100	43	2	2	47	47.0	148	43	2	1	46	31.1
32	14	4		18	56.3	52	22	11	2	35	67.3
48	3		3	6	12.5	76	100	10		110	144.7
30	15	2		17	56.7	46	11	5	5	21	45.7
48	14	2		16	33.3	30	15	2	2	19	63.3
61	27	2	2	31	50.8	68	10	14	14	38	55.9
86	23	5		28	32.6	61	24	16	3	43	70.5
30	1	7	7	15	50.0	98	57	13		70	71.4
30	1			1	3.3	79	3	2	7	12	15.2
97	31	2		33	34.0	43	18	3	1	22	51.2
30	2		4	6	20.0	125	89	9		98	77.8
89	22	6	4	32	36.0	120	54	8	12	74	61.7
68	7	2	7	16	23.5	100	66	6		72	72.0
31	7	2	2	11	35.5	62	44	12	17	73	117.7
40	8	2	3	13	32.5	100	97	2		99	99.0
120	64	8	4	76	63.3	100	89	10		99	99.0
87	18	7	2	27	31.0	30	6		3	11	36.7
72	13	3	1	17	23.6	116	41	2		43	37.1
82	39	6	2	47	57.3	120	74	7	3	84	70.0
(3) IL-3+G-CSF						40	11	3	2	16	40.0
Blast cell colony size	Number of secondary colonies				Replating efficiency(%)	36	51	7		58	161.1
	GM	GEMM	bl	Total		36	9	1		10	27.8
32	21	9		30	103.1	116	126	10		136	117.2
42	23	2	3	25	59.5	76	23	9	2	34	44.7
34	20	5		25	73.5	(4) IL-3+IL-1 β					
41	25	2		27	65.9	Blast cell colony size	Number of secondary colonies				Replating efficiency(%)
42	35	7		42	100.0		GM	GEMM	bl	Total	
51	31	9		40	78.4	34	13	5		18	52.9
45	26	1		27	60.0	71	38	5	1	44	62.0
42	19	17	8	44	104.8	47	25	4		29	61.7
41	1	1		2	4.9	35	9	7	4	20	57.1
51	24	10	2	36	70.6	37	12	1	1	14	37.8
35	11	3		14	40.0	40	12	3		15	37.5
90	56	4	2	62	68.9	31	7	1	1	9	29.0
39	12	9	6	27	69.2	14	3	1		4	28.6
38	9	3	2	14	36.8	35	10	2		12	34.3
79	33	6	1	40	50.6	48	24	5		29	60.4
55	32	3		35	63.6	46	24	8	3	35	76.1
50	10	4	2	16	32.0	83	24	8		32	38.6
41	36	2	1	39	95.1	37	19	2		21	56.8
134	87	12	2	101	75.4	31	1	1	1	2	6.5
44	24	16	3	43	97.7	36	1	1	1	3	8.3
109	38	2		40	36.7	46	11	1		12	26.1
105	30	2	1	33	31.4	56	19	6	2	27	48.2
36	9	6		15	41.7	61	24	4		28	45.9
30	17	1		18	60.0	49	31	11		42	85.7
95	10	1		11	11.6	31	3	4	2	9	29.0
87	45	2	2	49	56.3	74	40	6	2	48	64.9
94	46	3		49	52.1	64	30	2		32	50.0
52	42	1		43	82.7	42	10	8	7	25	59.5
63	28	5	1	34	54.0	96	55	4	5	64	66.7
38	11	6	15	32	84.2	70	7	8	1	16	22.9
100	69	8	9	86	86.0	108	54	11	8	73	67.6
77	30	5		35	45.5	70	24	6		30	42.9
43	21	8		29	67.4	77	30	4		34	44.2
73	73	11		84	115.1	54	15	3	4	22	40.7
64	79	1		80	125.0	71	2	1		3	4.2
						42	9	13	12	34	81.0
						30	8	3	2	13	43.3
						30	15	5		20	66.7
						72	48	10	1	59	81.9

Spleen cells of 5-FU treated mice, $1 \times 10^6/\text{ml}$, were cultured in the presence of IL-3, IL-3+IL-6, IL-3+G-CSF and IL-3+IL-1 β . From day 5 to day 10, newly appearing Bl, whose constituting cells were ranged between 30 and 150, were picked, and then replated in the presence of WEHI-3 CM and Epo.

Table 2 Comparison of self-renewal capacity of blast cell colonies supported by IL-3, IL-3 plus IL-6, IL-3 plus G-CSF and IL-3 plus IL-1 β

Stimulus	Blast cell colonies with secondary GEMM colonies	Blast cell colonies with secondary GEMM and blast cell colonies	P value
IL-3	39	21	0.5955
IL-3+IL-6	44	22	0.5858
IL-3+G-CSF	35	16	0.5758
IL-3+IL-1 β	34	18	0.5931

On day 12 of the secondary culture, secondary colony types including GEMM and BI were analyzed. According to the formula shown in "Materials and Methods", the p values and self-renewal probabilities were calculated in each group.

Table 3 Comparison of self-renewal capacity of blast cell colonies supported by IL-3 and IL-3 plus G-CSF

	Blast cell colony size (X)									Total		
	30≤X<100			100≤X<200			200≤X<400					
	A	B	P value	A	B	P value	A	B	P value	A	B	P value
IL-3	58	27	0.5777	39	6	0.5209	16	0	not calculated	113	33	0.5432
IL-3+G-CSF	48	23	0.5808	33	8	0.5346	23	8	0.5532	104	39	0.5584

BI were supported by IL-3 and IL-3+G-CSF. New BI, which appeared daily, were picked, and then replated.

A : Blast cell colonies with secondary GEMM colonies, B : Blast cell colonies with secondary GEMM and blast cell colonies.

production of the 2nd BI was ascribed to the self-renewal characteristic of stem cells. According to their calculation formula of the probabilities of self-renewal, p values were calculated (Table 2). These values were similar to the 0.589 that had been reported by Nakahata et al⁸⁾, whose work was done using the combination of pokeweed mitogen-stimulated spleen cell-conditioned medium (PWM-SCM) + Epo as cytokines. The addition of the so-called synergistic factors for dormant stem cells did not remarkably modify the self-renewal probabilities of the BI progenitors.

2. 2nd BI progenitor production

As we described previously^{12~14)}, the synergistic factors such as IL-6, G-CSF and IL-1 could shorten the G₀ periods of dormant hemopoietic stem cells and enhanced the BI formation in the earlier period of the culture. Also we reported that the synergistic factors could increase the frequencies of GEMM progenitor cells in constituting cells of BI¹⁴⁾. The true mechanism of this phenomenon was unclear, but there is a possibility that the synergistic factors might increase the 2nd dormant blast cells, thus producing a higher number of multipotential GEMM progenitors during the BI formation. In order to show that possibility,

Fig. 1 Size distributions of multipotential blast cell colonies with or without secondary blast cell colony progenitors. Spleen cells of 5-FU treated mice, $1 \times 10^6/\text{ml}$, were cultured in the presence of IL-3 and IL-3 + G-CSF. From day 5 to day 10, newly appearing Bl were picked, and then replated in the presence of WEHI-3 CM and Epo.

Fig. 2 Productive number of secondary blast cell colony progenitors. Spleen cells of 5-FU treated mice, $1 \times 10^6/\text{ml}$, were cultured in the presence of IL-3 and IL-3 + G-CSF. From day 5 to day 10, newly appearing Bl were picked, and then replated in the presence of WEHI-3 CM and Epo.

Table 4 Comparison of frequency of secondary blast cell colonies in the presence of IL-3 and IL-3 plus G-CSF

Blast cell colony size	Secondary blast cell colonies/Blast cell colony with secondary GEMM colonies	
	IL-3	IL-3+G-CSF
$30 \leq \text{size} \leq 100$	1.63	2.96
$100 < \text{size}$	0.17	1.10
Total	0.90	1.93

Absolute number of 2nd Bl progenitors was divided by the number of primary multipotential Bl.

Bl were grown in the presence of IL-3 and IL-3 + G-CSF from the spleen cells of 5-FU-treated mice. Bl were found daily, and were picked up and replated in the secondary culture. **Table 3** shows the frequencies of 2nd GEMM progenitors in the Bl constituting cells. Both the absolute number of the 2nd GEMM progenitors and the percentages of GEMM progenitors in Bl-forming cells were higher in the group supported by the combination of IL-3 and G-CSF than in that supported by IL-3 alone, independent of the Bl sizes.

The size distributions of the multipotential Bl are shown in **Fig. 1**. There was a tendency for the constituting cells in IL-3-supported Bl not to contain 2nd Bl progenitors when they grew in number to more than 150 cells. Comparatively, the combination of IL-3 and G-CSF kept the colony-constituting cells immature and supported larger Bl formations with the ability to produce 2nd Bl progenitors.

As shown in **Fig. 2**, IL-3-supported Bl usually produced 1 to 4 2nd Bl progenitors and 7 at most; however, IL-3 + G-CSF-supported Bl sometimes produced more than 5 2nd Bl progenitors. The total number of 2nd Bl progenitors divided by that of the initial multipotential Bl showed how many 2nd stem cells were produced by one initial stem cell (**Table 4**). The numbers were different among the cytokine groups and among the sizes of primary Bl, but there was a tendency for Bl forming cells supported by the combinations of IL-3 and G-CSF to produce higher number of 2nd stem cells reaching around 2 in total.

Discussion

The hemopoietic stem cells possess biologically important characteristics, such as multipotentiality, dormancy in the cell cycle and a self-renewal capacity. A lot of research has focused on these characteristics and the influence of cytokines. Nakahata et al⁸⁾ developed an in vitro Bl formation system and using this Bl assay they succeeded in calculating the self-renewal probability of the murine hemopoietic stem cells, which was found to be 0.589.

Recently several cytokines have been introduced and some of them were found to have some influence on the hemopoietic stem cells, such as shortening the G_0 phase of the cell cycle and expanding them both in vitro and in vivo. The synergistic factors like IL-6^{12,15)}, G-CSF¹³⁾, IL-1¹⁴⁾, IL-11¹⁶⁾ and SCF^{17,18)} enhanced the IL-3-dependent proliferation of the

hemopoietic stem cells and the mechanism was ascribed to the shortening of the G_0 phase. We found that these synergistic factors produced B1 with a higher number of 2nd multipotential GEMM progenitors. The mechanism was unclear, but there remains the possibility that those synergistic factors affect their self-renewal process, resulting in a higher production of 2nd B1 progenitors and GEMM progenitors.

In this paper, we examined the replating experiments of the B1-constituting cells and calculated the probability of self-renewal, according to the protocol reported by Nakahata et al⁸⁾. The results showed no differences in p values in the cases of IL-3, IL-3 + IL-6, IL-3 + G-CSF and IL-3 + IL-1. To our surprise, p values were narrowly fixed between 0.576 and 0.596 in the presence of one of three synergistic factors. Our hypothesis that synergistic factors could modify both proliferative character and decision-making process to self-renew of hemopoietic stem cells was not deliberate, and then two biological events might be totally different. However, when precisely examined, the colony size distribution of B1 that could produce 2nd B1 progenitors was different between the IL-3 and IL-3 + G-CSF group. The addition of G-CSF produced larger B1 with an ability to produce 2nd B1 progenitors. One possibility was that G-CSF kept the self-renewal process of the stem cells for a longer period of cell divisions. Again, there was a difference between IL-3-supported and IL-3 + G-CSF-supported B1 and the total number of 2nd B1 progenitors. The latter produced approximately double the number. This phenomenon might also be ascribed to the above-mentioned possibility.

Many investigators have reported the expansion system of murine and human hemopoietic stem cells using a combination of several cytokines. They documented a large scale expansion judging from the in vitro colony assays, HPP-CFC assay and in vivo CFU-S assay. Bodine et al⁹⁾ also reported the hemopoietic stem cell expansion system and they estimated the ratio by use of the in vivo competitive repopulation assay, which was done on day 35 and day 120 after transplantation of the expanded materials. Their data showed that the cytokines like IL-3 + IL-6, IL-3 + G-CSF, IL-4 + IL-6 and IL-3 + G-CSF + IL-6 expanded around 5 times more CFU-S than IL-3 alone after short term liquid cultures. However, according to their competitive repopulation assay, the expanded population stimulated by IL-3 + G-CSF and IL-3 + IL-6 repopulated around 65% of the overall hemopoiesis, while the control IL-3-stimulated population occupied 35%, the former having two times more reconstituting ability.

Other expanded population stimulated by IL-3 + G-CSF + IL-6, or IL-4 + IL-6 could occupy only 20 to 30% of the hemopoiesis. This had not been anticipated. There were differences in the expansion rates of hemopoietic stem cells under the two assay systems, but the competitive repopulation assay has several advantages, such as being an in vivo assay and being able to estimate the long-term-reconstitution ability of stem cells. Their data told us that the short term liquid culture of stem cells in the presence of IL-3 + IL-6 and IL-3 + G-CSF could expand reconstituting stem cells around two fold, which is a similar expansion value to our net product of 2nd B1 progenitors.

To further study the self-renewal process and expansion ratio of the hemopoietic stem

cells, it is important to establish the exact and short-term-assayable system of the stem cells. Several surface markers have been developed to differentiate immature progenitor cells and stem cells from the majority of mature hemopoietic cells, but it is still difficult to separate stem cells from other progenitor cells. Until we get such special markers, we cannot help depending on the in vitro colony assay and in vivo competitive assay.

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